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Mechanisms of metal resistance in plants: aluminum and heavy metals

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Abstract

Plants have evolved sophisticated mechanisms to deal with toxic levels of metals in the soil. In this paper, an overview of recent progress with regards to understanding fundamental molecular and physiological mechanisms underlying plant resistance to both aluminum (Al) and heavy metals is presented. The discussion of plant Al resistance will focus on recent advances in our understanding of a mechanism based on Al exclusion from the root apex, which is facilitated by Al-activated exudation of organic acid anions. The consideration of heavy metal resistance will focus on research into a metal hyperaccumulating plant species, the Zn/Cd hyperaccumulator, *Thlaspi caerulescens*, as an example for plant heavy metal research. Based on the specific cases considered in this paper, it appears that quite different strategies are used for Al and heavy metal resistance. For Al, our current understanding of a resistance mechanism based on excluding soil-borne Al from the root apex is presented. For heavy metals, a totally different strategy based on extreme tolerance and metal hyperaccumulation is described for a hyperaccumulator plant species that has evolved on naturally metalliferous soils. The reason these two strategies are the focus of this paper is that, currently, they are the best understood mechanisms of metal resistance in terrestrial plants. However, it is likely that other mechanisms of Al and/or heavy metal resistance are also operating in certain plant species, and there may be common features shared for dealing with Al and heavy resistance. Future research may uncover a number of novel metal resistance mechanisms in plants. Certainly the complex genetics of Al resistance in some crop plant species, such as rice and maize, suggests that a number of presently unidentified mechanisms are part of an overall strategy of metal resistance in crop plants.

Introduction

The molecular and physiological basis for crop plant interactions with the environment has attracted considerable interest in recent years. Within the general area of plant responses to the environment, one research area that has been the focus for many laboratories deals with the underlying mechanisms and strategies plants employ to tolerate potentially toxic levels of metals in the soil. A significant portion of this type of research has dealt with mechanisms that plants employ to deal with toxic levels of soil aluminum (Al) when cultivated on the significant areas of acid soils throughout the world. The focus of the research on plant Al toxicity has been to ultimately identify the genes conferring Al resistance, in order to assist in the development of crops that will be better suited for growth on acid soils. Another important area of metal resistance research involves molecular and physiological mechanisms of plant heavy metal resistance. One reason for increased interest in plant heavy metal interactions has been the recent attention on the use of plants to remediate heavy metal contaminated soils. Interest in this concept, termed phytoremediation, has in turn been driven partly by the growing awareness by the scientific community of the existence of a number of metal hyperaccumulating plant species. In a num-

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ber of laboratories, metal hyperaccumulator plants are currently being studied to gain a better understanding of molecular mechanisms that confer heavy metal hyperaccumulation and extreme metal resistance in plants.

In this paper, plant mechanisms of Al and heavy metal resistance will be compared and contrasted. The discussion of Al resistance mechanisms will deal with recent work by a number of laboratories on mechanisms of Al exclusion from the root apex in crop species that depend on Al-activated exudation of organic acid anions from the root tip. For the section on plant heavy metal resistance mechanisms, we will focus on metal hyperaccumulators as a dramatic example of tolerance to high levels of toxic metals in the soil, as well as the ability to accumulate those metals to extremely high levels in the shoot. For our examination of heavy metal hyperaccumulation, our laboratory's molecular and physiological investigations of the Zn/Cd hyperaccumulator, *Thlaspi caerulescens*, will be used as an example.

Plant Al resistance

As aluminum (Al) is the most abundant metal and third most abundant element in the earth's crust, plants have evolved in a soil environment where the roots are exposed to potentially high levels of aluminum. Fortunately, phytotoxic forms of Al are relatively insoluble at alkaline, neutral or mildly acidic soil pH values. However, at soil pH values at or below 5, the rhizotoxic Al species, Al^{3+}, is solubilized into the soil solution, inhibiting root growth and function and thus reducing crop yields. Acid soils limit agricultural productivity in many regions of the world. Approximately 30% of the world's total land area consist of acid soils, and it has been estimated that over 50% of the world's potentially arable lands are acidic (von Uexküll and Mutert, 1995). Acid soils limit the growth of crops in many developing countries where food production is critical. Furthermore, in developed countries such as the United States, high-input farming practices such as the extensive use of ammonia fertilizers are causing further acidification of agricultural soils. The addition of lime to acid soils can help to correct soil acidity, and this is a common agronomic practice in developed countries. However, the cost of purchasing and transporting lime to the farm precludes liming as an effective strategy for low-income farmers, and liming is not effective in correcting subsoil acidity. Therefore, considerable effort has been put into developing crop genotypes expressing increased Al resistance. For nearly 100 years, breeding programs have been effective in producing Al-resistant crop varieties, in particular for cereals such as wheat and maize (see, for example, Beckmann, 1976). Recently, there also has been a focus on molecular and physiological mechanisms of Al resistance. One of the primary goals of this type of research is the identification of Al resistance genes that can then be used via biotechnology to increase Al resistance in crop species for which significant natural variation in this trait does not exist.

Physiology of Al resistance

Two classes of mechanisms have been proposed to account for Al resistance: mechanisms that allow the plant to tolerate Al accumulation in the symplasm (Al tolerance), and those which exclude Al from the root apex (Al exclusion) (Delhaize and Ryan, 1995; Kochian, 1995; Kochian and Jones, 1997). There have been many different mechanisms proposed for Al tolerance and exclusion in the literature, with little evidence supporting most of these hypotheses. However, recent experimental evidence has been presented supporting the role of organic acid anion exudation from the root apex as a major mechanism of Al exclusion. The root tip is the site where Al resistance genes must act to control resistance mechanisms, as this is the site of Al toxicity (Ryan et al., 1993; Sivaguru and Horst, 1998). In a number of plant species (e.g., wheat, maize, buckwheat, rye, taro, snapbean) it has been shown that Al resistance by exclusion appears to be mediated by Al-activated release of organic acid anions such as malate, oxalate, or citrate, which chelate Al^{3+} in the rhizosphere and prevent its entry into the root apex (Ma et al., 2001).

When one considers all of the evidence in support of Al-induced organic acid release from the root as a *bona fide* Al resistance mechanism in plants, a very strong case in support of this hypothesis can be made. These findings include: (1) a strong correlation between Al resistance and Al-induced organic acid anion release in a range of different plant species (Delhaize et al., 1993a,b; Ma and Miyasaka, 1998; Ma et al., 1997a,b; Miyasaka et al., 1991; Pellet et al., 1995; Ryan et al., 1995a; Zheng et al., 1998); (2) an excellent correlation between the degree of Al resistance and magnitude of Al-induced root malate release in different wheat genotypes varying in Al resistance (ranging

from Al-sensitive to very Al-resistant) (Papernik et al., 2000; Ryan et al., 1995b); (3) the addition of organic acids (malate, citrate or oxalate) to root bathing solutions ameliorates Al toxicity in Al-sensitive varieties (see, for example, Delhaize et al., 1993b; Pellet et al., 1997; Zheng et al., 1998); (4) complexes of Al with di- and tricarboxylic organic acids are not transported across membranes or absorbed by roots (Akeson and Munns, 1989; Shi and Haug, 1990); (5) Al-induced malate release genetically cosegregates with Al resistance and Al exclusion from the wheat root apex (Delhaize et al., 1993a,b); (6) the rapid release of organic acid anions is consistent with the time frame for the onset of Al resistance (see, for example, Ryan et al., 1995a); (7) over-expression of the bacterial citrate synthase gene in tobacco and papaya resulted in increased citrate levels in roots, increased citrate exudation, and a significant increase in Al resistance (de la Fuente et al., 1997); and (8) an Al-gated anion channel has been identified in protoplasts isolated from the root apex of Al-resistant wheat and maize, which is a good candidate to be the transport system facilitating Al-induced organic acid anion release (Kollmeier et al., 2001; Pineros and Kochian, 2000; Ryan et al., 1997; Zhang et al., 2000).

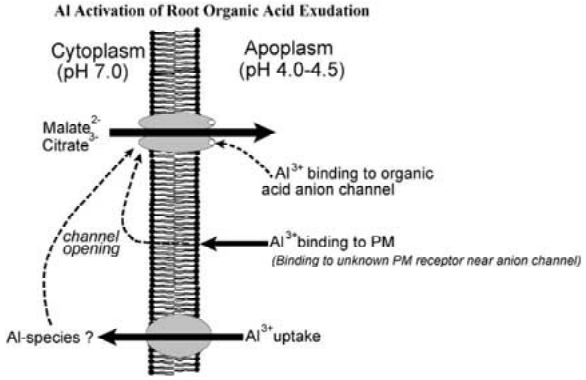


Figure 1. Possible mechanisms of Al-activated organic acid anion release involved in Al resistance via Al exclusion from the root apex. In this model, Al activation of an anion channel is proposed to be central to this resistance mechanism. This can be accomplished by: (1) the binding of extracellular Al^{3+} directly to the channel, which effects opening; (2) Al^{3+} binds to an unknown receptor in the plasma membrane, and mediates channel opening through a membrane-localized signal pathway; and (3) Al^{3+} enters the cell and triggers opening through a signal transduction cascade that involves both cytoplasmic and plasma membrane components.

The role of anion channels in the release of organic acid anions by roots

The numerous physiological studies investigating Al resistance mechanisms based on Al-activated organic acid anion exudation have provided strong evidence that the transport of organic acid anions across the root-cell plasma membrane and not organic acid synthesis is the key, rate limiting step in this process. In studies on Al-resistant and -sensitive genotypes of both wheat and rye it has been clearly shown that Al exposure only stimulates organic acid anion release from the root apex and has no effect on root apical organic acid concentrations. This stimulation occurs primarily in the resistant genotypes (Delhaize et al., 1993b; Li et al., 2000; Ryan et al., 1995, 2001). Furthermore, the Al-activated organic acid anion exudation continues for many hours without significant changes in any key enzymes involved in organic acid synthesis (PEP carboxylase, malate dehydrogenase, isocitrate dehydrogenase, citrate synthase). Based on studies such as these, it is widely accepted that Al is activating a plasma membrane organic acid anion transporter, and this transporter plays a central role in Al resistance (Delhaize and Ryan, 1995; Kochian, 1995; Kochian and Jones, 1997; Ryan et al., 2001).

Organic acids in the cytoplasm (pH 7) will be largely deprotonated and exist as anions, such that the Al-activated system involved in organic acid anion release and plant Al resistance likely involves an anion transporter. The thermodynamics of both inorganic and organic anion transport across the root-cell plasma membrane are such that there is a large outwardly directed electrochemical gradient for anions. Therefore, activation (i.e., opening) of plasma membrane anion channels will result in a large anion efflux, and it is likely that anion channels (permeable to organic acid anions) will constitute the transport mechanism via which Al-induced organic acid anion exudation occurs. As mentioned above, Ryan and co-workers have used the patch clamp technique on protoplasts isolated from the root apex of the Al-resistant wheat isoline expressing *Alt1* to identify a novel Al^{3+}-activated anion channel that could be the release pathway for malate exudation involved in Al resistance (Ryan et al., 1997).

More recently, they showed that this channel mediates malate transport in wheat roots and is inhibited by compounds that block both members of the *CLC* and *ABC* gene families of ion transporters (Zhang et al., 2001). They also showed that although this Al-activated anion current occurs in protoplasts from the root apex of both Al-resistant and -sensitive genotypes, the current occurred much more frequently,

exhibited a greater anion flux, and remained active longer in protoplasts isolated from the Al-resistant isoline (Zhang et al., 2001). These findings indicate that the same Al resistance machinery appears to operate in both Al-resistant and -sensitive genotypes, but the system is more active or abundant in the Al-resistant plants. As shown in Figure 1, there are three possible scenarios for Al regulating this anion channel that mediates organic acid anion efflux: (1) Al^{3+} binds directly to the channel and effects opening; (2) Al^{3+} binds to an unknown receptor in the plasma membrane, and mediates channel opening through a membrane-localized signal pathway; and (3) Al^{3+} enters the cell and triggers opening through a signal transduction cascade that involves both cytoplasmic and plasma membrane components.

Recent work from our lab and from the labs of Horst and Hedrich have identified an Al-gated anion channel in the plasma membrane of protoplasts isolated from Al-resistant maize, where Al is excluded from the root tip via Al-induced citrate release (Kollmeier et al., 2001; Pineros and Kochian, 2001). In the work by Kollmeier and colleagues, it was shown that this Al-activated channel mediates the transport of citrate, and is more active in root tip cells from Al-resistant *versus* Al-sensitive maize. In the work by Pineros and Kochian the potentially important result that Al-activation of the anion channel was observed in excised patches of the plasma membrane was presented. This finding indicates that the Al responsive machinery is localized to the plasma membrane, and either directly involves gating of the anion channel by Al or acts via a closely associated membrane receptor. These findings suggest that research should be directed to both plasma membrane organic acid anion transporters as well as regulatory proteins which may be closely associated with these transporters, as candidates for Al resistance genes.

Genetics and molecular biology of Al resistance

Significant variation for Al resistance is well known in many plant species and has led to a number of studies of inheritance for Al resistance (Foy, 1988). Wheat has been a widely studied crop species with regards to the genetics of Al resistance, and in wheat Al resistance is often monogenic (Camargo, 1984; Delhaize et al., 1993a; Kerridge and Kronstad, 1968; Riede and Anderson, 1996; Somers and Gustafson, 1995). However, there is also evidence to suggest that in certain very Al-resistant wheat cultivars, this trait is controlled by two genes (Berzonsky, 1992; Camargo, 1981). In our laboratories, a recent genetic analysis of Al resistance in barley (Tang et al., 2000) and sorghum (Magalhaes, Garvin, and Kochian, unpublished results) indicates that as in wheat, Al resistance appears to be a genetically simple trait. However, in certain other grain crop species, such as maize and rice, Al resistance appears to be a more complex character. The findings from several laboratories have indicated that in maize, Al resistance is conferred by multiple genes (Magnavaca et al., 1987; Sawazaki and Furlani, 1986); recent work on Al resistance in rice indicates a similar level of genetic complexity (Nguyen et al., 2001; Wu et al., 2000).

Al-inducible resistance genes

Many researchers have assumed that expression of Al resistance genes is induced by Al exposure, and this assumption has been the driving force for several studies in wheat aimed at cloning Al resistance genes. However, the considerable body of physiological information concerning Al resistance in wheat, which involves Al-activation of root apical malate exudation, suggests that Al resistance is not inducible. In wheat, all of the biochemical machinery for root Al exclusion via malate release appears to be in place before exposure to Al, and Al exposure appears to trigger this response at the level of protein activity rather than at the level of gene expression. Thus, attempts to clone Al-inducible Al resistance genes have identified stress response genes that are induced equally well in Al-resistant and -sensitive genotypes, usually well after the phenotypic expression of resistance is seen (see, for example, Richards et al., 1994; Snowden and Gardner, 1993).

In contrast to wheat there appears to be a different pattern of Al-activated organic acid anion release in certain other plant species, suggesting that Al resistance can also be an inducible process. In Al-resistant genotypes of rye and *Cassia tora*, there is a lag of up to 12 h between Al exposure and organic acid anion release, and the rate of organic acid anion release continues to increase upon continued exposure to Al (Li et al., 2000; Ma, 2000; Ma et al., 1997b). Based on these observations, the authors have speculated that Al induction of resistance genes occurs. Our own work on Al resistance in maize and sorghum strongly suggests that Al resistance is inducible in both of these crop species. In our laboratory, it was shown that Al resistance in maize involves Al activation of organic acid

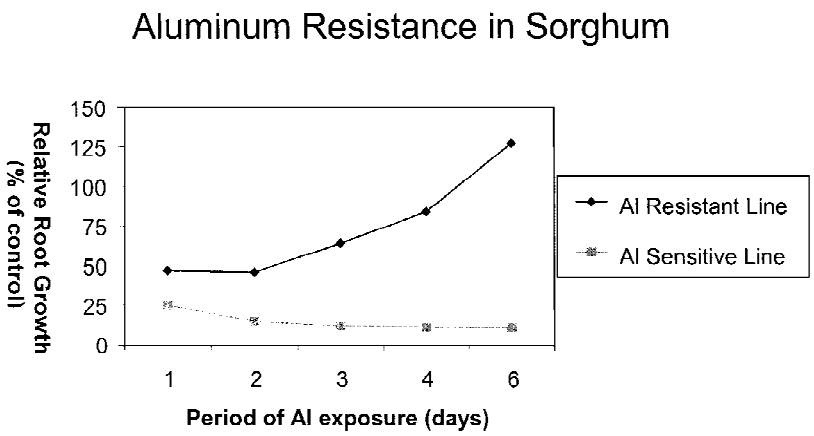


Figure 2. Influence of Al exposure (39 μM Al^{3+} activity in full nutrient solution) on root growth in Al-resistant and -sensitive parental lines of sorghum. Root growth is presented as per cent of control root growth measured in the absence of Al.

anion release (citrate) and Al exclusion from the root apex (Pellet et al., 1995). Al exposure rapidly triggers a low level of citrate exudation localized to the root apex of Al-resistant but not Al-sensitive maize genotypes. As shown in Pellet et al. (1995), upon continued Al exposure, the rate of citrate exudation increases continuously over the first 48 h of Al exposure. After 48 h the rate of citrate release levels off to a constant high rate. These physiologically based experiments suggest there is a constitutive level of Al resistance in maize; superimposed upon this is a resistance mechanism that is activated by Al exposure and could involve Al-induced expression of resistance genes.

In our recent work with sorghum, it was found that Al resistance is due to Al exclusion from the root apex mediated by Al-activated citrate release. As shown in Figure 2, continued exposure to Al in hydroponic media over a 6-day period induces an increasing level of Al resistance in the resistant parent, as measured by root growth in Al compared to control (−Al) plants. These findings indicate that as in maize, Al resistance appears to be Al-inducible in sorghum.

A primary goal for the ongoing Al resistance research in a number of laboratories is the isolation and characterization of Al resistance genes. This will be important both in gaining a complete understanding of this potentially complex trait, and also for use in improving the Al resistance of a number of crop species. Based on what is already known about the physiology and genetics of Al resistance, several different approaches are being taken by researchers attempting to isolate Al resistance genes. First, the recent identification of a major Al resistance mechanism involving Al-activation of organic acid anion efflux has provided several targets for candidate genes, including root plasma membrane organic acid anion transporters as well as other proteins that may interact with these transporters. As physiological and molecular genetic research on Al resistance in rice and sorghum moves forward, it may be possible to use map-based cloning approaches to identify Al resistance genes in these two model plant species. Finally, the possibility that resistance genes may be Al-inducible in some crop plant species suggests that genomics-based approaches may provide an additional avenue for the cloning of Al resistance genes.

Heavy metal hyperaccumulation and resistance

Terrestrial plants have evolved sophisticated strategies for the acquisition of relatively unavailable micronutrients such as Zn, Mn, Cu, Fe and Ni from the soil. As these essential micronutrients are also highly reactive and potentially toxic to plants, micronutrient uptake, transport and accumulation is by necessity highly coordinated and regulated. Because many micronutrients are also heavy metals, contamination of the soil environment with heavy metals is in reality the accumulation of high levels of either essential micronutrients (Zn, Mn, Cu, Ni), or metals that can act as micronutrient analogues (such as Cd, Pb, or Hg). Recently, progress has been made in elucidating the molecular and physiological mechanisms of plant micronutrient/heavy metal accumulation and homeostasis. There are a number of labs throughout the world

conducting research on plant heavy metal accumulation and resistance using metal hyperaccumulating plant species as model plants. The goal of this type of research is to provide the basic understanding and molecular tools that ultimately can be used to develop improved plant species for the remediation of metal-contaminated soils.

Contamination of soils with heavy metals is a serious worldwide problem both for human health and agriculture (Gairola et al., 1992; Mazess and Barden, 1991; Ryan et al., 1982). Cleanup of hazardous wastes by the currently used engineering-based technologies has been estimated to cost $400 billion dollars in the U.S. alone (Salt et al., 1995). Recently, there has been considerable interest in the use of terrestrial plants as an alternative, 'green technology' for the phytoremediation of surface soils contaminated with toxic heavy metals (Chaney, 1983; Cunningham and Ow, 1996; Raskin et al., 1997; Salt et al., 1995, 1998). A major factor behind the recent interest in phytoremediation of metal polluted soils has been the growing awareness by the scientific community of the existence of a number of metal hyperaccumulating plant species. Over 200 terrestrial species have been reported that are endemic to metalliferous soils and can tolerate and accumulate high levels of heavy metals such as Zn, Cd, Cu, and Ni in their shoots. These plants were coined hyperaccumulators by Brooks et al. (1977). The existence of these interesting metal hyperaccumulator species suggests that the genetic potential exists for phytoremediation to be successful. Most of these hyperaccumulator species, however, are small and slow growing, and because they produce limited shoot biomass their potential for large-scale decontamination of polluted soils is limited (Ebbs et al., 1997). Transferring the genes expressing the hyperaccumulating phenotype to higher shoot biomass-producing plants has been suggested as an avenue for enhancing the potential of phytoremediation as a viable commercial technology (Brown et al., 1995a). Progress towards this goal, however, has been hindered by a lack of understanding of the basic molecular, biochemical and physiological mechanisms involved in heavy metal hyperaccumulation.

One of the best known metal hyperaccumulators is *Thlaspi caerulescens*, which is a member of the Brassicaceae family and a Cd/Zn hyperaccumulator. Certain ecotypes of *Thlaspi caerulescens* have been shown to accumulate and tolerate up to 3000 μg g^{-1} DW Cd in the shoots (typical shoot levels are between 0.1 and 10 μg g^{-1} DW) and 40 000 μg g^{-1} DW Zn (normal foliar Zn levels for hydroponically grown plants are around 100–200 μg g^{-1} DW, while 30 μg g^{-1} DW is considered adequate) (Brown et al., 1995a,b). Additionally, certain ecotypes of *T. caerulescens* have been reported to accumulate high levels of other metals, including Ni and Co (Baker, 1981; Baker and Brooks, 1989; Brown et al., 1995b). The unique physiology of heavy metal transport and resistance in *Thlaspi caerulescens* makes it a very interesting experimental system for basic research aimed at elucidating plant mechanisms of heavy metal hyperaccumulation.

Physiology of Zn^{2+} transport in hyperaccumulator and non-accumulator species of Thlaspi

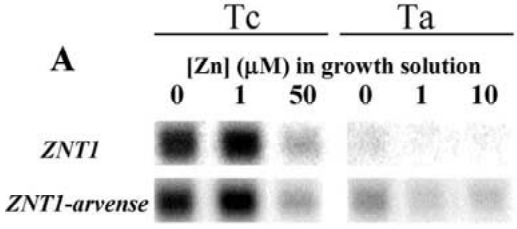
We initially conducted physiological studies that focused on the use of radiotracer flux techniques ($^{65}Zn^{2+}$) to characterize Zn transport and compartmentation in *Thlaspi caerulescens* and a related non-accumulator, *Thlaspi arvense*. These studies indicated that a number of Zn/Cd transport sites in the root and shoot contribute to the hyperaccumulation trait in *T. caerulescens*. As detailed in Lasat et al. (1996, 1998) these include: (1) a stimulated metal influx across the root-cell plasma membrane; (2) reduced metal sequestration in the root vacuole; (3) increased xylem-localized metal loading into xylem and subsequent translocation to the shoot; and (4) stimulated metal influx across the leaf cell plasma membrane and enhanced storage in the leaf vacuole. The stimulated Zn/Cd influx across the root-cell plasma membrane was studied in the greatest detail and it was shown that root Zn absorption is mediated by a saturable, high affinity Zn^{2+} transporter while the concentration-dependent kinetics for Cd influx were linear and non-saturating. The saturating Zn influx system had a similar affinity for Zn^{2+} in the two *Thlaspi* species (K_m for root Zn^{2+} uptake was 6 and 8 μM, in *T. caerulescens* and *T. arvense*, respectively). However, there was a 5-fold larger V_{max} for root Zn uptake in *T. caerulescens* compared with *T. arvense* (Lasat et al., 1996). While root Cd influx followed linear, first-order concentration dependent kinetics in the two *Thlaspi* species, it was 2–3-fold higher in *T. caerulescens*. These findings suggest that particularly the increased saturable Zn uptake in *T. caerulescens* could be due to a higher density of Zn transporters in the root-cell plasma membrane.

Molecular basis of zinc hyperaccumulation in Thlaspi caerulescens

A molecular characterization of plant heavy metal hyperaccumulation was initiated by cloning a Zn transporter cDNA from *T. caerulescens* via functional complementation in yeast. The *Saccharomyces cerevisiae* mutant, ZHY3, defective in the high and low affinity Zn transporters, ZRT1 and ZRT2, has a much higher Zn requirement for growth than the parental wild type yeast (Zhao and Eide, 1997). The ZHY3 strain was transformed with a *T. caerulescens* cDNA library constructed in the yeast expression vector, pFL61. Screening of 350 000 yeast transformants for growth on low Zn media resulted in the identification of *ZNT1* (for Zn transporter) (see Pence et al., 2000).

Expression of *ZNT1* in ZHY3 restored growth on low Zn media to that of the parental wild type yeast. The predicted open reading frame for *ZNT1* is 379 amino acids in length and demonstrates significant sequence identity with the *Arabidopsis* genes *ZIP4* and *IRT1*, which encode putative Zn and Fe transporters, respectively (Eide et al., 1996; Grotz et al., 1998) and are members of the ZIP family of micronutrient transport proteins (Eng et al., 1998; Guerinot, 2000). ZNT1 shares the structural features exhibited by other members of this family, including eight putative transmembrane domains and a highly hydrophilic cytoplasmic region predicted to reside between transmembrane domains three and four. This putative cytoplasmic domain contains a series of histidine repeats, which may define a metal binding region for the transporter. The similarities in predicted amino acid sequence and protein structure between ZNT1 and other members of the ZIP family suggest that ZNT1 is an integral membrane protein that mediates Zn^{2+} transport across a cell membrane.

To test the hypothesis that ZNT1 is a Zn transport protein, *ZNT1* was expressed in yeast (ZHY3) for radiotracer ($^{65}Zn^{2+}$ and $^{109}Cd^{2+}$) flux experiments that were used to determine the concentration dependent kinetics of Zn^{2+} and Cd^{2+} influx mediated by *ZNT1* in ZHY3. We found that ZNT1 mediated saturable Zn uptake that conformed to Michaelis–Menten kinetics with a K_m of 7.5 μM, as well as low affinity Cd^{2+} influx in yeast that follows first-order (linear) transport kinetics (Pence et al., 2000). The kinetic properties for Zn^{2+} and Cd^{2+} uptake mediated by *ZNT1* in yeast are very similar to what we have previously seen for Zn^{2+} and Cd^{2+} uptake in *T. caerulescens* roots (Lasat et al., 1996). These results are consistent with the hypothesis that *ZNT1* encodes a root plasma membrane Zn^{2+}/Cd^{2+} transporter.



B Root Zn transport kinetic parameters

4	6	5	2	2	2	K_m
244	271	76	80	43	43	V_{max}

Figure 3. Influence of plant Zn status on *ZNT1* expression and root Zn^{2+} uptake. Seedlings of *T. caerulescens* (lanes labeled Tc) and *T. arvense* (lanes labeled Ta) were grown in nutrient solution containing 0, 1 and 10 or 50 μM Zn. Root RNA was isolated for Northern analysis and hybridized to gene-specific probes for *ZNT1* in *T. caerulescens* and for the *ZNT1* homolog in *T. arvense*. Also, ^{65}Zn flux studies were conducted to determine the concentration-dependent kinetics of root Zn uptake in the *Thlaspi* seedlings. The K_m and V_{max} for high affinity ^{65}Zn uptake are presented below the Northern.

A 5-fold increase in the V_{max} for root Zn^{2+} influx in *T. caerulescens* compared with *T. arvense* in an earlier study led us to speculate that there are a greater number of Zn transporters per unit area of root-cell plasma membrane in the hyperaccumulator (Lasat et al., 1996). In order to test this further, seedlings of both *Thlaspi* species were grown under Zn deficient (0 μM), 'normal' Zn conditions (1 μM) and high Zn conditions (50 μM Zn for *T. caerulescens* and 10 μM Zn for *T. arvense*). Then, the concentration-dependent kinetics of root Zn^{2+} influx and *ZNT1* Northern analysis using RNA isolated from roots and shoots of both *Thlaspi* species grown under all three Zn treatments was conducted (Figure 3 and Pence et al, 2000). When gene specific probes for *ZNT1* or its homolog from *T. arvense* were used, we found that *ZNT1* transcript abundance was dramatically higher in roots and shoots of *T. caerulescens* grown under 'normal' Zn (1 μM) and Zn-deficient conditions (0 μM) as compared with *T. arvense*. This is consistent with the hypothesis that Zn hyperaccumulation in *T. caerulescens* is due, in part, to increased expression of Zn transporters in the root and shoot.

The root Zn transport data and *ZNT1* transcript abundance as a function of seedling Zn status provides

Table 1. ZIP family micronutrient transporters cloned in *Thlaspi caerulescens*

Thlaspi caerulescens	*Arabidopsis homolog*	% identity between *T. caerulescens* and *A. thaliana*	Length in *T. caerulescens*
ZNT1	*ZIP4*(U95973)	88	Full length
ZNT2	*ZIP2*(AF033536)	88	Mising 28 bp at 5′ end
ZNT3	*ZIP3*(AF033537)	87	Missing 200 bp at 5′ end
ZNT4	*IRT3*(AAF27669)	86	Full length
ZNT5	*ZIP5*(AAB71447)	86	Full length

ZIP1, 7 and *9* have been cloned in *Arabidopsis* and are being used to isolate *T. caerulescens* homologs.

insights into the regulation of Zn transporters in hyperaccumulator and nonaccumulator plants. A close correlation between *ZNT1* expression (Figure 3A) and the V_{max} for root Zn^{2+} influx (Figure 3B) was found in both *Thlaspi* species. In *T. arvense*, growth on 'normal' (1 μM) or high Zn (10 μM) had no effect on the low level of root *ZNT1* expression or the small root Zn^{2+} influx that was observed (V_{max} of 43 nmol gm h). Only when *T. arvense* plants were made Zn-deficient, was the moderate increase in *ZNT1* expression and root Zn^{2+} influx seen (increase in V_{max} to 80 nmol gm^{-1} h^{-1}). Quantification of root transcript abundance from the data in the Northern blot indicated that Zn deficiency caused a 2-fold increase in *T. arvense* mRNA abundance, which correlates with the 2-fold enhancement of V_{max}.

The response of root Zn uptake to changes in plant Zn status in *T. caerulescens* were found to be qualitatively similar to the responses in *T. arvense* when seedlings were grown in a wide range of Zn concentrations in the nutrient solution (0–500 μM Zn). That is, *T. caerulescens* seedlings grown in 0 and 1 μM Zn had very high level of *ZNT1* expression as well as a considerably larger V_{max} for root Zn^{2+} influx (V_{max} values of 244 and 271) in comparison with *T. arvense*. However, when *T. caerulescens* seedlings were grown on levels of Zn ranging from 50 to 500 μM (which are comparable to levels of available Zn^{2+} for Zn-contaminated soils), a significant down-regulation in *ZNT1* expression and reduction in root Zn^{2+} uptake were observed (V_{max} reduced to 76 nmol gm^{-1} h^{-1} and a 6-fold reduction in root mRNA abundance). Although growth on high Zn reduced *ZNT1* expression and Zn uptake in *T. caerulescens*, they were still 4- and 2-fold higher, respectively, than in Zn-sufficient *T. arvense*. Thus, it appears that an alteration in the regulation of Zn transport by Zn status, and not a constitutive increase in Zn transporter gene expression, plays a role in Zn hyperaccumulation.

If a Zn responsive regulatory scheme similar to that in yeast exists in higher plants, how might it be altered to cause the enhanced Zn transporter gene expression and Zn hyperaccumulation observed in *T. caerulescens*? One possibility involves a mutation in a putative Zn responsive transcriptional activator, which would alter Zn-dependent down-regulation of *ZNT1* expression. Such a mutation in a Zn-responsive transcriptional activator, *ZAP1*, has been isolated in yeast (Zhao and Eide, 1997; Zhao et al., 1998). The semi-dominant mutant allele, $ZAP1\text{-}1^{up}$, results from a substitution of a serine for a cysteine residue in the N terminal region, and causes high level of expression of the yeast Zn transporters under Zn replete conditions. Thus, specific alterations in a Zn-responsive transcriptional activator or Zn- responsive elements in transporter gene promoters possibly play an important role in heavy metal hyperaccumulation in *T. caerulescens*.

Involvement of multiple micronutrient transporter genes in metal hyperaccumulation

The results presented above suggest that *ZNT1* could play an important role in the root and leaf metal transport processes resulting in hyperaccumulation, based on the correlations between the magnitude of root Zn^{2+} influx and *ZNT1* expression. We have found using *in situ* RT-PCR techniques that *ZNT1* expression is localized to the root cortex, further supporting a role for this transporter in root metal absorption from the soil (Letham et al., 2000). Information from the *Arabidopsis* genome sequencing efforts as well as advances from other laboratories (Eng et al., 1998; Grotz et al., 1999) have shown that there are a large number of members of the ZIP/IRT family of micronutrient transporters in *Arabidopsis* including 14 possible ZIPs and three IRTs. Indications of a similarly large transport gene family in *T. caerulescens*

came from Southern analysis of *ZNT1* under low stringency, which yielded a complex pattern indicative of *ZNT1* hybridization to other members of the ZIP family in *Thlaspi* (data not shown). As it appears that *ZNT1* plays an important role in heavy metal hyperaccumulation in *T. caerulescens*, the existence of other closely related genes certainly raises the possibility that other members of this family also are important to metal hyperaccumulation. To get a start on this area of research we have cloned four other *T. caerulescens* homologs of the *ZIP* gene family and are in the process of isolating three more; these are summarized in Table 1.

From the findings presented in Figure 3, we wondered whether in addition to *ZNT1*, other *T. caerulescens* genes involved in metal hyperaccumulation may be expressed to much higher levels in *T. caerulescens* compared with the nonaccumulator, *T. arvense*. Northern analysis has been conducted with some of the other ZIP genes as well as other heavy metal-related genes. It was found that at least two other members of the *ZIP* family of metal transporters in *T. caerulescens* as well as homologs of *Arabidopsis* metallothionein and N*ramp* genes were expressed to much higher levels in *T. caerulescens* compared with *T. arvense*. It should be noted that when the blot was probed with three different *AtNramp* genes, only a homolog to *AtNramp2* exhibited increased transcript abundance, suggesting that overexpression is specific to particular genes in individual heavy metal transporter gene families.

The *ZIP* micronutrient transporter gene family in higher plants could be regulated by a Zn-dependent transcriptional activator as has been shown for yeast, where the transcription factor *ZAP1* interacts with Zn-responsive elements in the promoters of the *ZIP* homologs, *ZRT1-3* (Zhao and Eide, 1997; Zhao et al., 1998). The results described above indicating that at least one *Nramp* gene also is up regulated in *T. caerulescens* suggests that in plants, a Zn-dependent factor could also regulate expression of genes from other, 'non-*ZIP*' transporter gene families. Further support for this possibility comes from our recent observation that expression of at least one *ZAT* homolog (a putative vacuolar Zn transporter from the Cation Diffusion Facilitator [CDF] family of micronutrient transporters) is up regulated in *T. caerulescens*. Also, work from Dr. Eide's laboratory has shown that in yeast, *ZAP1* can regulate the expression of a number of different genes in a Zn-dependent manner, including the *ZRC1* gene which encodes a vacuolar Zn/heavy metal transporter from the CDF family of transporters (Lyons et al., 2000). Taken together, all of these pieces of evidence provide circumstantial support for a model of heavy metal hyperaccumulation in *T. caerulescens* that involves alterations in the 'normal' regulation of micronutrient homeostasis that occurs in non-accumulator plant species.

Conclusions

In this paper, we have described specific mechanisms that plants employ to deal with toxic levels of aluminum and heavy metals in the soil. Based on an analysis of the current literature, quite different strategies appear to be used for Al and heavy metal resistance. For Al, most of the evidence points to an Al resistance mechanism based on exclusion of Al from the root apex. This involves Al-activation of a transporter localized to the root-cell plasma membrane that mediates the release of organic acid anions into the rhizosphere. These organic anions complex and detoxify Al^{3+} in the soil. For heavy metals, a totally different strategy based on extreme tolerance and metal hyperaccumulation was described for a hyperaccumulator plant species that has evolved on naturally metalliferous soils. The reason these two strategies were the focus of this paper was that currently, they are the best understood mechanisms of metal resistance in terrestrial plants. However, it is likely that other mechanisms of Al and/or heavy metal resistance are also operating in certain plant species, and there may be common features shared for dealing with Al and heavy metals. For example, a second Al resistance mechanism has recently been described in hydrangea and buckwheat that involves internal detoxification of accumulated Al by organic acids (citrate and oxalate) (Ma et al., 1997; 1998). This mechanism allows these plant species to accumulate Al in their leaves to quite high levels in hydrangea (3000 ppm) and moderately high levels in buckwheat (450 ppm). In comparison, plant species such as wheat, which employ the Al exclusion mechanism described in this paper, accumulate less than 50 ppm Al in their leaves (Ma, 2000). It is likely that future research on plant metal resistance will uncover novel mechanisms of metal tolerance that currently have not been described.

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